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Investigations on the Growth of *Rhodococcus rubra* in Relation to the Formation of Stable Biological Foams

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Abstract–The growth characteristics of a foam-forming species, *Rhodococcus rubra* were studied on different substrates. The basic medium contained Czapek (3.34%), yeast extract (0.2%), potassium dihydrogen phosphate (0.12%), dipotassium hydrogen phosphate (0.25%) and ammonium chloride (0.1%). This was supplemented with varying concentrations of glucose (0-2%). The same basic medium was also used to examine the growth of *R. rubra* in combination with varying concentration of n-hexadecane (0.0-0.5%) as a source of energy while varying the concentration of ammonium chloride in the range 1-3 g l^{-1}. Studies based on determining the biomass concentration, the surface activity related to the cell suspensions and measuring the variations in broth pH revealed that glucose encouraged the growth of *R. rubra*, compared to the control. However, increasing the glucose concentration from 0.1 to 2.0% had no further effect on growth. The surface activity of the cell suspensions increased with increasing glucose concentration. Results similar to glucose were exhibited by the addition of n-hexadecane, suggesting same degree of growth among different concentrations with higher surface activity increasing with increase in substrate concentration. Results have also shown that the pH of all the culture broths decreased as the ammonium chloride concentration increased, suggesting that there was a production of hydrogen ions during the course of its metabolism.

Key words: Wastewater Treatment, Stable Foam, *Rhodococcus rubra*, Surface Activity

INTRODUCTION

The activated sludge process, which is based on microbial reactions, suffers from two potential biologically based operational problems: sludge bulking and stable foam formation. Bulking causes poor settling of sludge and can, therefore, produce low quality effluent. The formation of stable biological foams can also produce low quality effluent, which is a world-wide problem first reported in 1969 [Anon, 1969]. Since then, several workers [Greenfield et al., 1985; Soddel and Seviour, 1995; Khan and Forster, 1988, 1991; Iwahori et al., 1997; Sunairi et al., 1997; Rossetti et al., 1997] have reported the occurrence of stable foams when specific filamentous species dominate the mixed liquor suspended solids. In the main, the filamentous species involved are actinomycetes; *Nocardia*, *Microthrix* or *Rhodococcus*, and it has been shown that low temperature foams are more likely to be caused by *Rhodococcus* species [Soddel and Seviour, 1995]. Appreciable amounts of oil and grease in the incoming sewage have previously been associated with foam formation [Greenfield et al., 1985]. Foam forming actinomycetes, which appear to be highly hydrophobic [Sunairi et al., 1997; Stratton et al., 1998], concentrating at air/water interface are able to metabolize lipids and hydrocarbons that are not readily available to many other bacteria. For example, *Gordona* (formerly *Nocardia*) *amarae* utilizes hydrocarbons and produces surface-active compounds [Cairns et al., 1982; Takeda et al., 1992]. *Microthrix parvicella* has shown enhanced growth when oleate was used as a main carbon source, storing oleate during growth cycle [Slijkhuis et al., 1984], which results in imparting surface activity to the cells. *Rhodococcus spp.* also utilise hydrocarbons [Iwahori et al., 1995; Aburuwaida et al., 1991; Mercade et al., 1996] and produce biosurfactants [Khan and Forster, 1988; Aburuwaida et al., 1991; Mercade et al., 1996]. Another study [Khan and Forster, 1991] has revealed that *R. rubra* showed enhanced growth when it was grown on straight chain alkanes and on the corresponding carboxylic acids and that the degree of enhancement increased with chain length.

Foams in activated sludge plants are most certainly stabilised by surface-active compounds which are either excreted from the bacterial cells in the sludge or are components of the cell surfaces. However, there is no clear picture about the way in which these foam-forming species become dominant in the mixed liquors, particularly as they are such slow growing species. It could be an environmental effect, it could be due to nutritional imbalances or it could be the result of selective inhibition. Any information about any of these effects is a valuable addition to the central pool of knowledge about foam formation. Previous work [Khan and Forster, 1988] has examined the growth characteristics of one of the foaming species, *Rhodococcus rubra* grown on a basic medium, Czapek-Yeast Extract. This paper, therefore, reports an extension of this work which examined the effect on the growth and surface properties of *Rhodococcus rubra* when different supplements were added to this basic medium.

METHODS AND MATERIALS

Pure cultures of *Rhodococcus rubra* (supplied by the Pollution Research Unit UMIST) were maintained on Czapek media sup-

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Table 1. Growth media for *R. rubra*

Component	Concentration (%; w/v)	
Basic medium		
Czapek broth	3.34	
Yeast extract	0.20	
NH_4Cl	0.10	
K_2HPO_4	0.25	
KH_2PO_4	0.12	
Supplements		
1. Glucose	0.0-2.0	
2. n-Hexadecane/	n-hexadecane (%; v/v)	NH_4Cl (g l^{-1})
Ammonium chloride	0.0	1
	0.1	1
	0.1	2
	0.1	3
	0.5	2
	0.5	3

plemented with Yeast extract as agar slopes grown at 28 °C for 10-12 days. These were used to grow starter cultures which were then used as 0.3% v/v (7 days old) inocula in the subsequent growth studies.

A series of 500 ml conical flasks containing 200 ml medium was used for the growth studies. The compositions of the growth media used are given in Table 1. The basic medium (Czapek broth) contains: sodium nitrate (2.0 g l^{-1}), potassium chloride (0.5 g l^{-1}), magnesium glycero-phosphate (0.5 g l^{-1}), ferrous sulphate (0.01 g l^{-1}), potassium phosphate (0.35 g l^{-1}) and sucrose (30.0 g l^{-1}). All the flasks were incubated at 28 °C and at a shaking rate of 105±2 revolutions per minute. Biomass concentration, pH and surface tension of the cultures were measured daily.

Biomass concentrations were determined gravimetrically by filtration through membrane filters (0.45 μm pore size). The daily pH of the culture broth was measured at room temperature by a pH meter (Corning, model 7, USA). Surface tensions of the culture broth were measured with a torsion balance (White Elec. Inst. Co. Ltd.) by using the technique that has been described previously [Khan and Forster, 1988]. The average of at least five readings was reported.

RESULTS

Fig. 1 shows the effect of the glucose supplements on the growth of *Rhodococcus rubra*, and it is apparent that the addition of glucose brought about an increase in the amount of biomass. The glucose supplements also produced an increase in the rate of growth during the period 24 to 72 hours. However, the results demonstrate that increasing the glucose concentration from 0.1 to 2.0% (data for 2.0% not shown) did not have any further effect, either on the growth rate or on the ultimate biomass concentration. Fig. 2 shows the changes in the surface activity of the various culture broths. There was little difference in the pattern of variation in the control sample and that supplemented with 0.1% glucose. However, as the glucose concentration was increased, the differences became greater, with the surface tension of the broth supplemented with 2% glucose plateauing at about 42 dynes cm^{-1} compared with a value of about 55 dynes cm^{-1} for the control sample. The reason for the substantial drop in the surface tension, which occurred, between 24 and 48 hours is unknown, although a re-examination of Fig. 1 shows that this was the period of maximum growth-rate. The data contained in Fig. 3 show the variations in the broth pH values during the growth period. Although, the pH values of all the supplements (except 0.1% glucose) varied identically, there were quite distinct differences between those supplemented with the higher glucose concentrations (0.5, 1.0 and 2.0%) and the other samples.

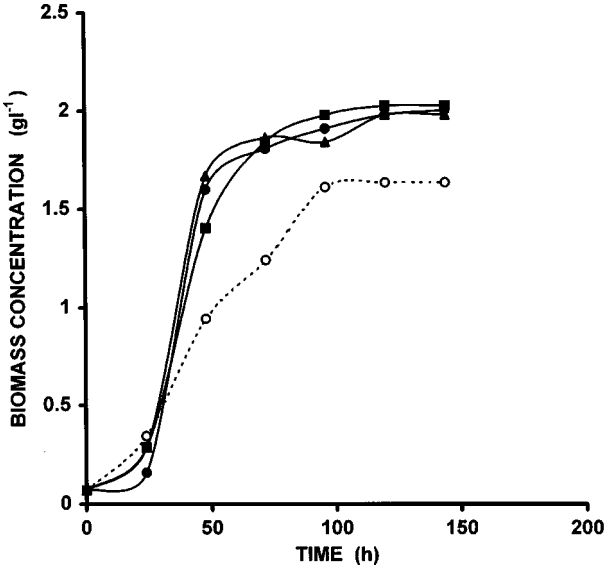

Fig. 1. Growth of *R. rubra* on media supplemented with different concentrations of glucose.
○: control, ■: 0.1% glucose, ▲: 0.5% glucose, ●: 1.0% glucose

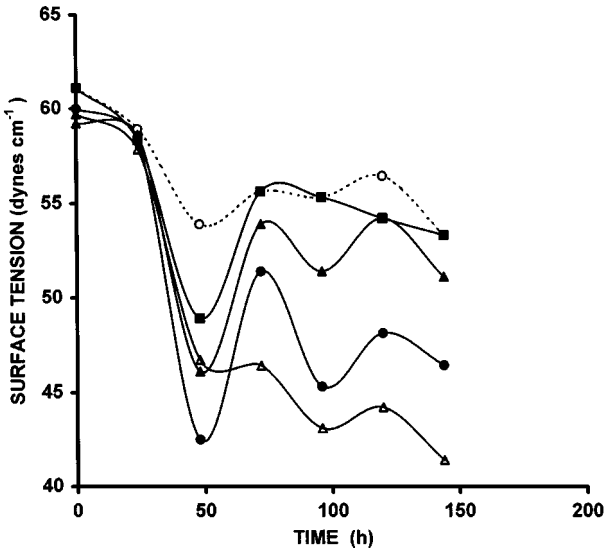

Fig. 2. Variations in the surface activity of *R. rubra* grown on media supplemented with different concentrations of glucose.
○: control, ■: 0.1% glucose, ▲: 0.5% glucose, ●: 1.0% glucose, △: 2.0% glucose.

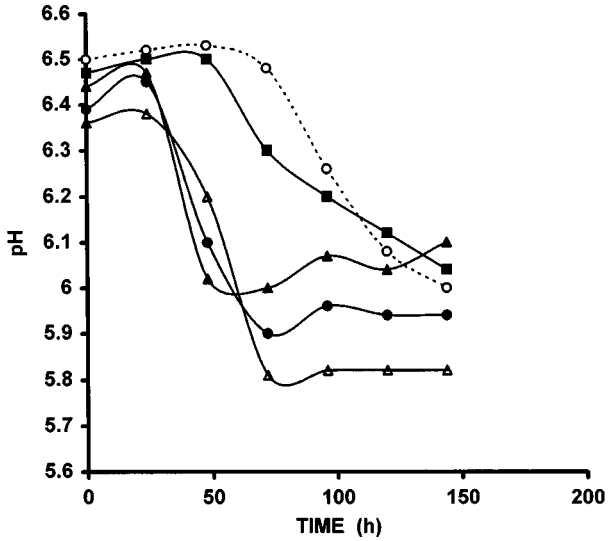

Fig. 3. pH variations during the growth of *R. rubra* on media supplemented with different concentrations of glucose.
○: control, ■: 0.1% glucose, ▲: 0.5% glucose, ●: 1.0% glucose, △: 2.0% glucose.

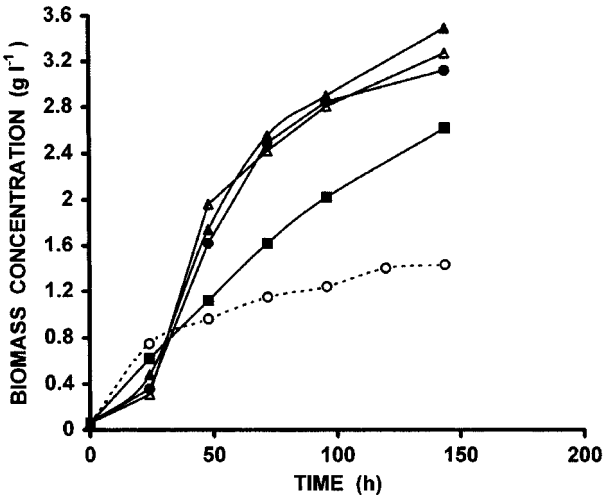

Fig. 4. Biomass variations during the growth of *R. rubra* on media supplemented with n-hexadecane and ammonium chloride.
○: control - Czapek/yeast extract media [5], ■: 1 g l^{-1} ammonium chloride: 0.0% n-hexadecane, ▲: 1 g l^{-1} ammonium chloride: 0.1% n-hexadecane, ●: 2 g l^{-1} ammonium chloride: 0.1% n-hexadecane, △: 3 g l^{-1} ammonium chloride: 0.1% n-hexadecane.

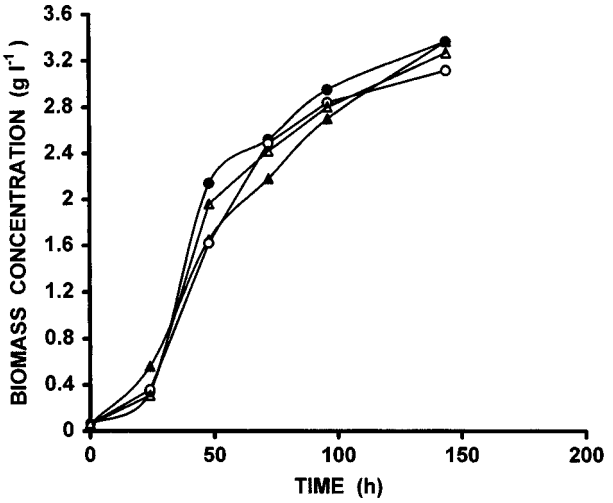

Fig. 5. Biomass variations during the growth of *R. rubra* on media supplemented with n-hexadecane and ammonium chloride.
●: 2 g l^{-1} ammonium chloride: 0.5% n-hexadecane, ○: 2 g l^{-1} ammonium chloride: 0.1% n-hexadecane, ▲: 3 g l^{-1} ammonium chloride: 0.5% n-hexadecane, △: 3 g l^{-1} ammonium chloride: 0.1% n-hexadecane.

These differences were most marked in the 24-72 hour period (the logarithmic and the early stationary phases). Previous work has shown that when *R. rubra* was grown on Czapek medium supplemented with nutrient broth or with starch, the pH increased with time. However, the pH was found to decrease with time when the Czapek medium was enriched with ammonium ions [Khan and Forster, 1996]. This decrease was attributed either to a release of intracellular acids as lysis occurred [Gray et al., 1984] or to an imbalance in the hydrogen ion concentration due to the metabolism of the ammonium ions [Macaskie, personal communication]. Khan and Forster [1996] also examined the effect of pH on the surface activity of the biosurfactant isolated from *R. rubra* and showed, that at pH values between 7 and 10, variations in pH had no effect on the surface activity.

As with all microbes, the growth of *R. rubra* is dependent upon the availability of degradable substrates. The n-alkanes, after oxidation to primary alcohols, form the corresponding fatty acids that are further degraded to lower chain fatty acids. These metabolites may influence the growth of this species. Therefore, the role of n-hexadecane was investigated. Figs. 4 and 5 show the growth of *R. rubra* in broths which had been supplemented with a straight chain alkane (n-hexadecane) and with varying concentration of ammonium chloride. In Fig. 4, the results have been compared with previously published data [Khan and Forster, 1991]. The results show that n-hexadecane clearly enhances the growth of *R. rubra*, compared with broths devoid of n-hexadecane, specifically Czapek broth supplemented with yeast extract [Khan and Forster, 1991]. An examination of Fig. 4 shows that increasing the concentration of ammonium chloride from 1 to 3 g l^{-1} had very little effect on the growth-rate or the ultimate biomass concentration. An examination of Fig. 5 shows that increasing the concentration of n-hexadecane from 0.1 to 0.5% did not significantly affect either the growth-rate or the ultimate biomass concentration. It was also observed that, during the first 24 hours, as n-hexadecane started to be metabolized by the cells, aggregates or clusters of the cells, in the form of long thread-like structures were formed. However, they appeared to be relatively weak structures as they broke down into individual cells when they were shaken vigorously.

Fig. 6 shows how the surface tension of the various broths varied during the growth of *R. rubra*. These data clearly show distinct features that are mainly related to the n-hexadecane concentrations. All the broths containing concentrations of 0.1% n-hexadecane behaved identically, but their pattern of behaviour was different from

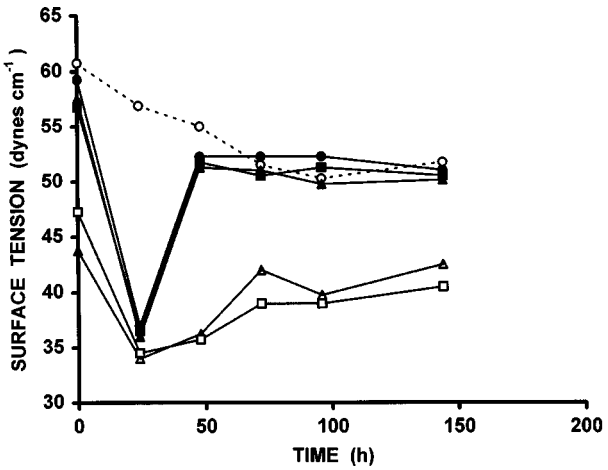

Fig. 6. Surface tension variations during the growth of *R. rubra* on media supplemented with n-hexadecane and ammonium chloride.
○: 1 g l^{-1} ammonium chloride: 0.0% n-hexadecane, ●: 1 g l^{-1} ammonium chloride: 0.1% n-hexadecane, ▲: 2 g l^{-1} ammonium chloride: 0.1% n-hexadecane, △: 2 g l^{-1} ammonium chloride: 0.5% n-hexadecane, ■: 3 g l^{-1} ammonium chloride: 0.1% n-hexadecane, □: 3 g l^{-1} ammonium chloride: 0.5% n-hexadecane.

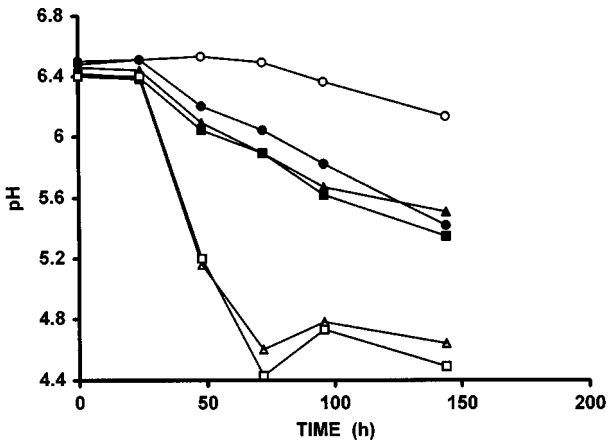

Fig. 7. pH variations during the growth of *R. rubra* on media supplemented with n-hexadecane and ammonium chloride.
○: 1 g l^{-1} ammonium chloride: 0.0% n-hexadecane, ●: 1 g l^{-1} ammonium chloride: 0.1% n-hexadecane, ▲: 2 g l^{-1} ammonium chloride: 0.1% n-hexadecane, △: 2 g l^{-1} ammonium chloride: 0.5% n-hexadecane, ■: 3 g l^{-1} ammonium chloride: 0.1% n-hexadecane, □: 3 g l^{-1} ammonium chloride: 0.5% n-hexadecane.

those of the control and the samples containing 0.5% of n-hexadecane. All the samples, except the control, showed an abrupt and marked decrease in surface tension during the first 24 hours. This was followed by an appreciable increase between 24 and 48 hours. This increase was, however, less marked in the broths containing 0.5% of n-hexadecane. Fig. 7 shows the variations in the pH values during the tests with n-hexadecane and ammonium chloride and demonstrates that, with this particular set of media, there were very distinct differences between the control sample, the samples containing n-hexadecane concentrations of 0.1% and those containing n-hexadecane concentrations of 0.5%. With the control sample and the samples containing the lower n-hexadecane concentration, the change in the pH values was steady and continuous. In the samples containing the higher n-hexadecane concentration,

Table 2. Growth and surface tension profile of *R. rubra*

Growth media	Cell growth		Surface tension* (dynes/cm) at	
	Specific growth rate, μ (hr^{-1})	Maximum biomass (g l^{-1})	24 hours	48 hours
• Sucrose (Czapek media)	0.9779	1.63	59.0	54.0
• Glucose				
0.0%	0.9779	1.63	59.0	54.0
0.1%	1.1685	2.02	58.5	48.7
0.5%	0.9704	1.98	58.0	46.5
1.0%	1.1860	2.00	58.0	42.2
2.0%	1.1312	1.96	46.5	47.0
• n-Hexadecane				
0.0% (+1.0 g/*l* NH_4Cl)	0.9958	2.61	57.0	56.0
0.1% (+1.0 g/*l* NH_4Cl)	1.0457	3.48	37.0	53.0
(+2.0 g/*l* NH_4Cl)	1.0971	3.11	36.5	52.6
(+3.0 g/*l* NH_4Cl)	1.0973	3.26	37.0	52.5
0.5% (+2.0 g/*l* NH_4Cl)	1.1549	3.36	33.2	41.5 at 72 hrs**
(+3.0 g/*l* NH_4Cl)	0.9948	3.36	34.5	39.0 at 72 hrs**

*During exponential growth - values of significance.
**Delayed effect due to higher concentration of n-hexadecane (0.5%).

there was an abrupt change in the 24 to 72 hours period after which the pH values remained more or less constant.

DISCUSSION

The established mechanism of the metabolism of glucose and sucrose in heterotrophs is well known. Glucose metabolizes through glycolysis and TCA cycle to produce energy and provide precursors for cell material. Growth of *R. rubra* on the glucose supplemented broth (Fig. 1) showed an increase in the biomass concentration in comparison to the control. Increasing the concentrations of glucose from 0.1 to 2.0% did not have any further effect either on the rate of growth or on the ultimate biomass concentration. The data in Table 2 substantiate this behaviour. The broth devoid of glucose (Czapek media) contains sucrose as a main carbon source. The specific growth rate of this medium was found to be 0.9779 hr^{-1}. The media containing 0.1 to 2.0% glucose showed an increase in the biomass; however, the ultimate biomass which is reported as a maximum biomass as a rough estimate in the present studies varied in the range 1.96 to 2.02 g l^{-1}, averaging at 1.99 g l^{-1}. The specific growth rate of glucose (Table 2) encompassed in the present studies also showed a less significant change, ranging from 0.9704 to 1.1860 hr^{-1}, averaging at a value of 1.1140 hr^{-1}. Glucose has been used previously as a non-substrate, growth-enhancing agent in both aerobic and anaerobic reactors [Catchpole and Cooper, 1972; Carliell et al., 1995; Mousa and Forster, 1998], and it is suggested that this may be its mode of action in the current study. The reason for this type of behaviour is not known. In contrast, Fig. 2 shows that the surface tension of broths containing 2.0% glucose was nearly 13 dynes cm^{-1} lower than the control containing no glucose. This quite marked decrease occurred between 24 and 48 hours in the growth cycle when the culture was in the logarithmic growth phase. The reasons for this and the oscillatory behaviour of the surface tension with growth time are not known, although they could be due to the formation or presence of metabolites during growth on glucose. This could also be due to the formation of some modified form of glycoplipids which were then metabolized later in the growth cycle [Mercade et al., 1996].

The higher concentrations of glucose (0.5-2.0%) caused a substantial drop in pH (Fig. 3) which followed a similar pattern to the surface tension changes. It has been reported previously that the pH of *R. rubra* culture increases as growth proceeds [Khan and Forster, 1988, 1996]. Although, the study made by Khan and Forster [1996] who used ammonium sulphate as a supplement in the growth media reported that the pH of the culture broth (Fig. 3) dropped during the exponential growth. The pH of a broth can be reduced by lysis of older cells [Gray et al., 1984] or by the production of CO_2 during the metabolism of glucose [Gottschalk et al., 1979]. Alternatively, as argued by Khan and Forster [1996], the metabolism of ammonium ions would result in an imbalance in the hydrogen ion concentration and a decrease in the pH.

The data in Figs. 4 and 5 show the growth of *R. rubra* cultures in broths supplemented with n-hexadecane at various concentrations of ammonium chloride. The data in Fig. 4 clearly demonstrate that more biomass was obtained in the n-hexadecane supplemented broths. The data in Fig. 4 further show that increasing the ammonium chloride concentration from 1 to 3 g l^{-1} had no effect on the growth-rate or the ultimate biomass concentration. The specific growth rate and the maximum biomass concentration data presented in Table 2 have substantiated this effect. The specific growth rate of 0.1% n-hexadecane broth (with 1.0% ammonia) was found to be 1.0457 hr^{-1} increased insignificantly to 1.0971 hr^{-1} when ammonia concentration was increased to 2.0 g l^{-1}. However, no further change in the specific growth rate was brought by 3.0 g l^{-1} ammonia, where the μ value remained unchanged (1.0973 hr^{-1}). Little variation (mainly a decreasing trend) was, however, found in the maximum biomass during the ammonia range 1-3 g l^{-1}. The variations in the maximum cell mass remained between 3.11-3.48 g l^{-1} averaging at 3.28 g cells/l for 0.1% n-hexadecane. Similarly, the data in Fig. 5 demonstrate that increasing the concentration of n-hexadecane from 0.1 to 0.5%, had a little effect on the growth-rate but no effect on the ultimate biomass concentration was found. The specific growth rate (Table 2) increased slightly from 1.0971 to 1.1549 hr^{-1} at 2.0 g l^{-1} ammonia concentration. However, increasing the concentration of ammonia to 3.0 g l^{-1} decreased the μ value to 0.9948 hr^{-1}. Overall, the maximum biomass was slightly increased with the increase in n-hexadecane concentration from 3.11 to 3.36 g l^{-1}. No further increase among the 0.5% n-hexadecane supplements was recorded as a function of ammonia. The effect of 0.1% n-hexadecane as a function of ammonia in Table 2 has suggested a slight increase in the μ values when the ammonia level was increased from 1 to 2 g l^{-1}; however, no change thereafter was brought by ammonia. This indicates the least role of ammonia in increasing the specific growth rate of *R. rubra*.

The data in Fig. 6 show that broths containing 0.1% n-hexadecane behave in a similar way and that broths containing 0.5% n-hexadecane also followed a similar trend. There was an abrupt decrease in the surface tension during the first 24 hours in the period before the cells had entered the exponential phase. This was followed by a sharp increase in the surface tension in the 24-48 hour period of growth. It has been observed that, about 6 hours after inoculation, the cells started sticking together and long thread-like structures, which were distinctly hydrophilic, were formed in the broth. These thread-like structures were present until the cells entered the logarithmic phase. This thread-bearing stage in broth with 0.5% n-hexadecane was found to be stretched to 48 hours compared to 24 hours in 0.1% of n-hexadecane. However, these thread-like structures were found to be weak and would break into individual cells upon vigorous shaking. After the disappearance of these structures, the surface tension of the cultures increased again. Ramsay et al. [1988] have observed similar structures when *Rhodococcus aurantiacus* was grown on n-hexadecane. However, no explanation to this effect was given in relation to surface tension except that during the thread-bearing stage, the cells were hydrophilic. Previously, Zajic et al. [1977] found that the pure linear hydrocarbons in the range of n-C_{12} to n-C_{17} were the preferred substrate for the growth of *Corynebacterium hydrocarboclatus*. Alkanes are metabolised to several intermediates before becoming accessible to the microbe. They are first converted to the corresponding alcohols and then to the corresponding carboxylic acids which are then degraded by the process of β-oxidation [Khan and Forster, 1991]. The decreases in surface tension during the first 24 hours could well be due to the formation of fatty acids that are potentially surface active. During the exponential growth, further de-

gradation would result in compounds that were less surface active. In hydrocarbon fermentation, the production of extracellular surface active or emulsifying compounds is effective in enhancing the availability of the insoluble substrates. If this is the case here, then the potent biosurfactants formed during the first 24 hours of growth, which reduced the surface tension markedly, have been utilized in bringing the insoluble n-hexadecane to a form which is more accessible (carboxylic acid) to *R. rubra*, then an increase in the surface tension could be expected [Neufeld and Zajic, 1984]. Furthermore, Cooper and Zajic [1980] have reported that the surface tension of medium chain fatty acids decreases with the length of the carbon chain down to C_{12} after which it starts to increase again. Khan and Forster [1996] later confirmed this behaviour with 0.1% mixtures of C_{10}-C_{17} carboxylic acids. The decrease and increase in surface tension in this current study is identical to earlier results suggesting the metabolism of n-hexadecane and the formation/presence of corresponding carboxylic acids as degraded metabolites. The variation in surface tension as a function of ammonia (1-3 g l^{-1}), on the other hand, was found to be minimal (Table 2) during the significant part of the growth (i.e., exponential growth). The minimum values of surface tension varied in the range 36.5-37.0 dynes/cm, whereas maximum values varied between 52.5-53.0 dynes/cm (Table 2).

Fig. 7 shows the pH variations that occurred during the growth of *R. rubra*. The variations shown by the control and the 0.1% supplemented broth were distinctly different from those containing 0.5% n-hexadecane concentration. The main conclusion that can be drawn from these results is that, although the metabolism of the ammonium ions will affect the pH (as discussed earlier), the presence of the n-hexadecane will also influence the pH due to the production of fatty acids. However, it is not possible to separate these two effects or to determine which was the more dominant.

CONCLUSIONS

This paper has examined the growth of *R. rubra* by using different concentrations of glucose, n-hexadecane and ammonium chloride as supplements. The biomass yield per unit mass of added carbon using the maximum biomass at the lowest concentration of the supplements, encompassed in the present studies, indicated that sucrose produced the lowest yield (0.116 g cells mass/g of sucrose carbon utilized). Glucose (0.1%) produced 5.05 g culture mass/g of glucose carbon utilized, whereas 0.1% n-hexadecane (with 2 g l^{-1} ammonia) yielded 3.66 g biomass/g of hexadecane carbon utilized. It is, therefore, apparent that the substrates more than 0.1% glucose and 0.1% n-hexadecane remained mainly unutilized. Probably, that is the reason why the cell mass among the different concentrations of glucose did not vary significantly with the increase in substrate concentration. In the case of 0.5% n-hexadecane, the surface tension values did not change significantly after 72 hours of growth due to the presence of non-metabolized substrate. The delayed disappearance of the long thread-like structures could also be attributed to this effect. The results have also shown that

- glucose encouraged the growth of *R. rubra*
- increasing the concentration of glucose in the range 0.1-2.0% had no further effect on growth
- the surface activity of the cell suspension increased with the glucose concentration
- n-hexadecane favoured growth of *R. rubra*
- same degree of growth exhibited in the range 0.1-0.5% of n-hexadecane
- surface activity of the *R. rubra* cells found dependent upon the substrate concentration, with surface activity increasing with the increase in n-hexadecane concentration
- ammonium chloride was found to be mainly responsible for decrease in the pH of the culture broth.

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